Perspectives in Science (2015) 4, 38–45

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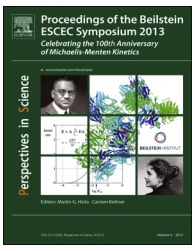

REVIEW

The accidental assignment of function in the tautomerase superfamily ☆

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Received 17 March 2014; accepted 8 December 2014
Available online 24 December 2014

KEYWORDS
Tautomerase;
Misassignment of function;
Functional diversity;
Structure-function relationship;
volution of enzymes

Abstract
Cg10062 from *Corynebacterium glutamicum* is a tautomerase superfamily member with the characteristic $\beta-\alpha-\beta$ fold and catalytic Pro-1. It is a *cis*-3-chloroacrylic acid dehalogenase (*cis*-CaaD) homologue with high sequence similarity (53%) that includes the six critical active site residues (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, and Glu-114). However, Cg10062 is a poor *cis*-CaaD: it has much lower catalytic efficiency and lacks isomer specificity. Two acetylene compounds (propiolate and 2-butynoate) and an allene (2,3-butadienote) were investigated as potential substrates for Cg10062. Cg10062 is a hydratase/decarboxylase using propiolate and *cis*-3-chloro- and 3-bromoacrylates, where malonate semialdehyde is the product of hydration and acetaldehyde is the product of decarboxylation. The two activities occur consecutively using the initial substrate. In contrast, 2-butynoate and 2,3-butadienote only undergo a hydration reaction with Cg10062 to afford acetoacetate. *cis*-CaaD does not function as a hydratase/decarboxylase using any of these substrates, yielding only the products of hydration. Cg10062 proceeds by direct hydration or covalent catalysis (using Pro-1) depending on the substrate. Direct hydration yields the hydration products and covalent catalysis yields the hydration and decarboxylation products. Cg10062 mutants shift the reaction toward one or the other mechanism. The observation that propiolate is the best substrate suggests that Cg10062 could be a hydratase/decarboxylase in a pathway that transforms an unknown acetylene compound to acetaldehyde via propiolate. The bifunctional activity of Cg10062 might also have implications for the evolution of the dehalogenase and decarboxylase activities in the tautomerase superfamily.

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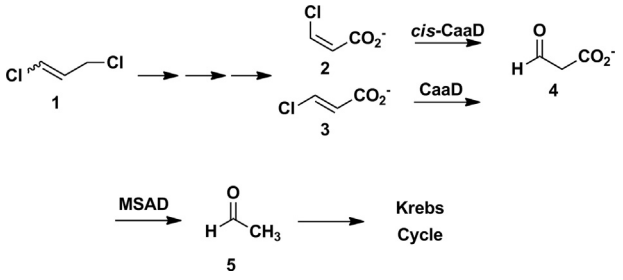
http://dx.doi.org/10.1016/j.pisc.2014.12.007

Contents

Introduction

cis-3-Chloroacrylic acid dehalogenase (*cis*-CaaD) and Cg10062, a *cis*-CaaD homologue in *Corynebacterium glutamicum*, are two closely related enzymes in the tautomerase superfamily (Poelarends et al., 2004a, 2008a, 2008b). The first one has a known function in a well-established catabolic pathway (Poelarends et al., 2004a). The second one does not have a known function or a genomic context that provides clues about possible functions (Poelarends et al., 2008a). However, our recent studies of *cis*-CaaD and Cg10062 with acetylene and allene substrates suggest a possible function and biological role for Cg10062. Cg10062 might also be representative of a common ancestral enzyme that diverged to give the separate dehalogenase and decarboxylase activities in the tautomerase superfamily (Poelarends et al., 2005; Almrud et al., 2005).

cis-CaaD catalyses the conversion of *cis*-3-chloroacrylic acid (**2**, Scheme 1) to malonate semialdehyde (**4**). The enzyme is a trimer where each monomer consists of 149 amino acids. It does not use coenzymes or metal ions to assist in the reaction. The enzyme is part of a degradative pathway for the nematicide 1,3-dichloropropene (**1**). In three enzyme-catalysed steps, the isomeric mixture of **1** is converted to the *cis*- and *trans*-isomers of 3-chloroacrylate (**2** and **3**, respectively). Isomer-specific dehalogenases (*cis*-CaaD and *trans*-3-chloroacrylic acid dehalogenase or CaaD) convert the respective isomers to **4**. Subsequently, malonate semialdehyde decarboxylase (MSAD) processes **4** to acetaldehyde (**5**), which is then channelled to the Krebs cycle (Poelarends et al., 1998).



Scheme 1

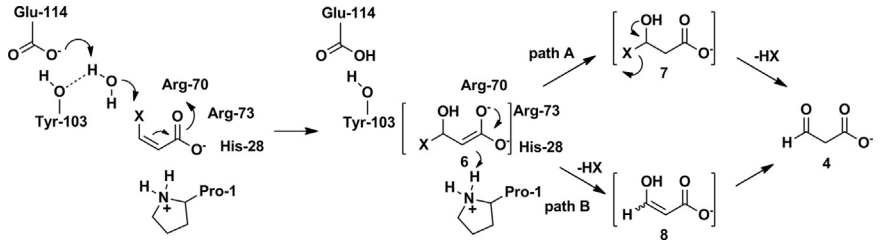
The tautomerase superfamily

One interesting feature of the 1,3-dichloropropene catabolic pathway is that three of the enzymes (*cis*-CaaD, CaaD, and MSAD) are tautomerase superfamily members. The tautomerase superfamily is a group of structurally homologous proteins characterized by a $\beta-\alpha-\beta$ building block and a catalytic amino-terminal proline (Poelarends et al., 2008b). Nature has used this building block to produce diverse structures and activities. Characterization of the individual members provides insight into how nature creates these activities and how divergent evolution proceeds.

The amino-terminal proline in the tautomerase superfamily can function as a general base catalyst or a general acid catalyst, depending on its pK_a value (Poelarends et al., 2008b). In several members, Pro-1 has a low pK_a value ($\sim$6.4) so that it functions as a general base catalyst (Stivers et al., 1996). In other members, the pK_a value is higher ($\sim$9.2) so that Pro-1 exists in the cationic form and functions as a general acid catalyst. In *cis*-CaaD, the pK_a value of Pro-1 is estimated to be 9.2, based on a pH rate profile (Poelarends et al., 2004b). More generally, the pK_a values for Pro-1 in tautomerase superfamily members are determined by direct titration using ^{15}N NMR spectroscopy and uniformly ^{15}N-labelled enzyme (Poelarends et al., 2008a). These experiments have not been carried out with *cis*-CaaD or Cg10062.

Characterization of *cis*-CaaD

Sequence analysis, mutagenesis and kinetic experiments, inhibition studies, and several crystal structures (with and without ligands) identified six key active site residues for *cis*-CaaD activity (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, and Glu-114) (de Jong et al., 2007; Guo et al., 2011). Based on the positions of these residues in crystal structures, a working hypothesis was formulated for the mechanism (Scheme 2). The substrate (*cis*-3-bromo or 3-chloroacrylate) is bound in the active site where three residues (His-28, Arg-70, and Arg-73) interact with the C-1 carboxylate group. These interactions bind and polarize the substrate to create a partial positive charge at C-3. The combination of Glu-114 and Tyr-103 activates a water molecule, which attacks at C-



Scheme 2

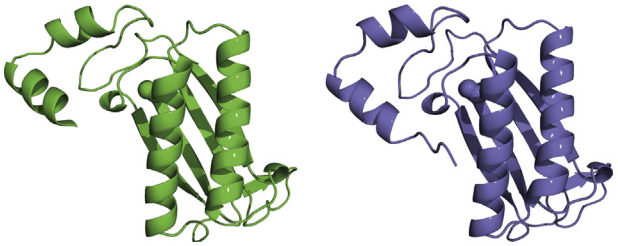


Figure 1 The Cg10062 monomer (left) and the *cis*-CaaD monomer (right) (PDB codes 3N4D and 2FLT, respectively). Both monomers (149 amino acids) show the signature $\beta-\alpha-\beta$ fold of the tautomerase superfamily where two $\beta-\alpha-\beta$ building blocks are covalently joined by a short linker. The catalytic amino-terminal proline is shown in space-filling form.

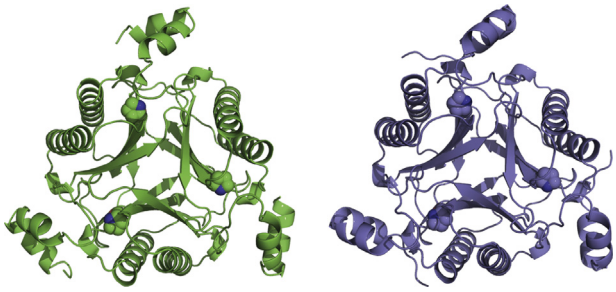


Figure 2 The Cg10062 trimer (left) and the *cis*-CaaD trimer (right) (PDB codes 3N4D and 2FLT, respectively). The catalytic Pro-1 is shown in space-filling form in each of the three active sites.

3 to form an *aci*-carboxylate species (**6**, Scheme 2). The species collapses by one of two paths to produce malonate semialdehyde (**4**). In path A, protonation at C-2 by Pro-1 produces the halohydrin **7**, which expels HX to yield **4**. In an alternate path B, an α,β-elimination takes place to afford enol **8**. Tautomerization of **8** produces **4**.

Characterization of Cg10062

In the course of a search for other *cis*-CaaD family members, we identified an enzyme in *C. glutamicum* with the gene designation Cg10062 (Poelarends et al., 2008a). The enzyme shares 34% sequence identity and 54% similarity with *cis*-CaaD and includes the six key residues required for *cis*-CaaD activity (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, and Glu-114). In addition, the monomers (Figure 1), trimers (Figure 2), and active sites (including the key residues, Figure 3) are superimposable (Guo et al., 2011). For these reasons, it was anticipated that Cg10062 would function as a *cis*-CaaD with the same mechanism and activity even though there did not appear to be a catabolic pathway for **1** in *C. glutamicum* (based on the genomic context). However, a comparison of the kinetic parameters for *cis*-CaaD and Cg10062 showed that **2** is a poor substrate for Cg10062 due to a high value of K_m (72,000 vs 200 μM) resulting in an overall k_{cat}/K_m of 14 $M^{-1}s^{-1}$ (1570-fold lower than that measured for *cis*-CaaD) (Poelarends et al., 2008a; Schroeder et al., 2012, 2013). In addition, Cg10062 processed the *trans* isomer, albeit poorly (0.8 $M^{-1}s^{-1}$ using **3**) (Poelarends et al., 2008a). This observation sharply contrasts with both CaaD and *cis*-CaaD, which are highly specific for their respective isomers.

Cg10062 is a hydratase/decarboxylase

It is frequently observed that two closely related enzymes do not have comparable levels of activity or even the same activities (Lu et al., 2011). There are generally three possible explanations for these observations. First, there could be subtle changes in the active sites that alter the positioning of the active site groups with regard to binding of the substrate and/or catalysis (Li et al., 2008). Second, there can be changes outside the active side where the sequence similarities are not as high. For example, a loop could move into the active site upon substrate binding in one enzyme, but not in the other (Schroeder et al., 2013). There could also be conformational dynamics originating outside the active site (Whittier et al., 2013). Finally, the substrate for one enzyme might just not be the substrate for the other enzyme.

Exploration of the first two possibilities did not provide an obvious explanation. The active site of Cg10062 might be more spacious than that of *cis*-CaaD, but there is not an apparent link between the spaciousness and the reduced *cis*-CaaD activity (Schroeder et al., 2013). There is also a 6-residue loop (Thr-32-Gly-33-Thr-34-Gln-35-His-36-Phe-37) that closes down on the active site in a covalently modified crystal structure of *cis*-CaaD (Robertson et al., 2009; Schroeder et al., 2013). The prolyl nitrogen is attached to (*R*)-2-hydroxypropanoate moiety, resulting from the enzyme's incubation with (*R*)-oxirane-2-carboxylate. In Cg10062, the loop consists of different residues (Ala-32-His-33-Ala-34-Pro-35-Lys-36-Tyr-37) and does not close in on the active site in a similarly covalently modified crystal structure. A mutational analysis of the loop residues by swapping those in *cis*-CaaD for the corresponding ones in Cg10062 was inconclusive (Schroeder

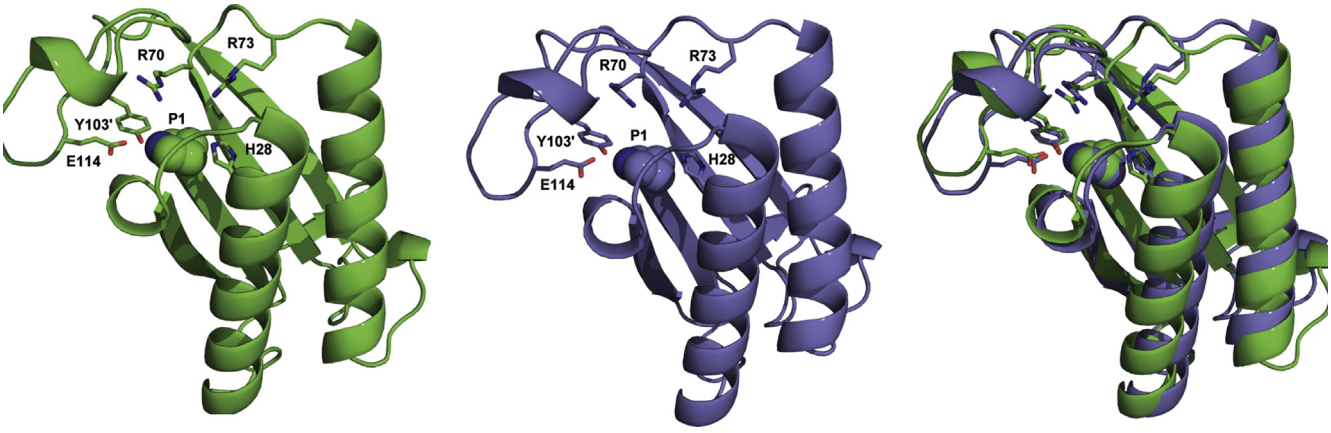
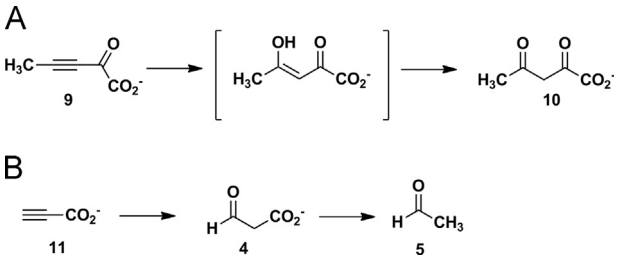
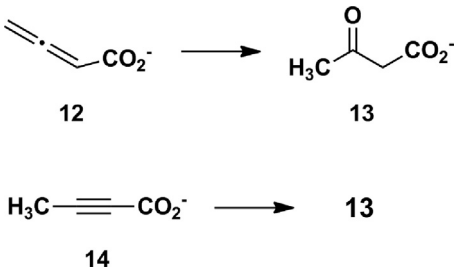


Figure 3 A comparison of the active sites of Cg10062 (left), *cis*-CaaD (centre), and an overlay (right) (PDB codes 3N4D and 2FLT). The catalytic residues (Pro-1, His-28, Arg-70, Arg-73, Tyr-103, Glu-114) are shown in Cg10062 and *cis*-CaaD. The overlay (right) shows how the actives sites of the two enzymes superimpose. The prime on Y103 indicates that this residue comes from a different monomer.



Scheme 3



Scheme 4

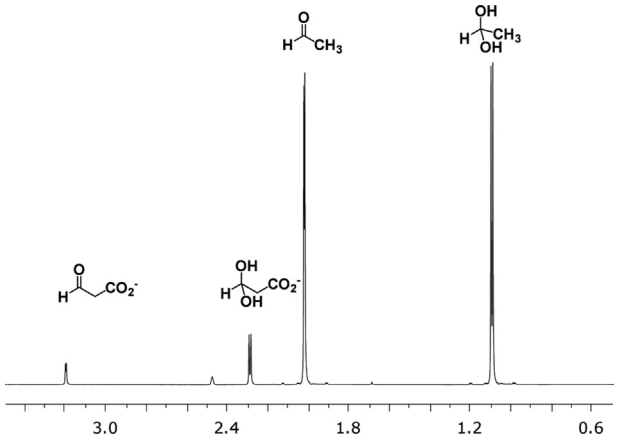


Figure 4 The ^{1}H NMR spectrum of the reaction mixture containing Cg10062 and **11**, recorded after 3 min. Analysis of the spectrum shows a mixture of **4** (~15%) and **5** (~85%). There is no further enzyme-catalysed decarboxylation of **4**. The unlabelled signal at 2.49 ppm corresponds to dimethyl sulfoxide-d_6, which is used as a lock signal.

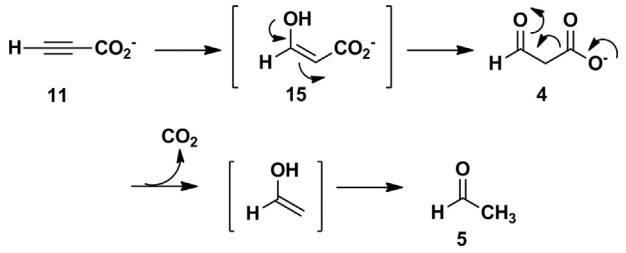
et al., 2013). The contribution of the *cis*-CaaD loop to binding and catalysis is not yet clear, and further work is necessary.

The third possibility provides the most satisfactory explanation. It might be that the 3-haloacrylates are not the biological substrates for Cg10062. Such a possibility was uncovered accidently when a halide-free solution of **4** was desired. Previous to this work, it was only possible to generate **4** using the *cis* or *trans* isomer of 3-haloacrylate (i.e., **2** or **3**) and the appropriate dehalogenase. It is known that Cg10062, *cis*-CaaD, and CaaD process the acetylene compound, 2-oxo-3-pentynoate (**9**, Scheme 3A), to acetopyruvate (**10**), presumably by the Michael addition of water across the triple bond and subsequent tautomerization (Poelarends et al., 2008a, 2008b). Hence, it was anticipated that *cis*-CaaD and Cg10062 might process the 3-carbon acetylene compound propiolate (**11**) to **4** (Scheme 3B). Indeed, **11** is a good substrate for *cis*-CaaD (k_{cat}~3.2 s^{-1}, K_m~500 μM, and k_{cat}/K_m~6.4 × 10^3 M^{-1} s^{-1}), but an even better one for Cg10062 (k_{cat}~6 s^{-1}, K_m~33 μM, and k_{cat}/K_m~1.8 × 10^5 M^{-1} s^{-1}).

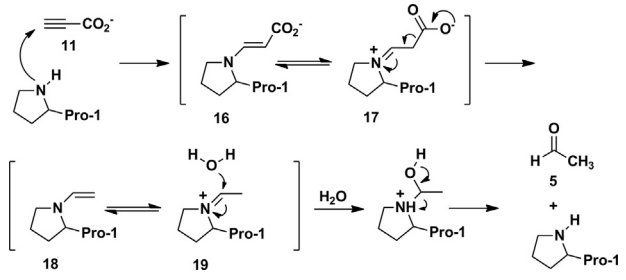
However, a ^{1}H NMR analysis showed that the product of the Cg10062-catalysed reaction is mostly acetaldehyde, **5**, (85%) along with a small amount of **4** (15%) (Figure 4). (The product of the *cis*-CaaD-catalysed reaction is **4**.) The reaction was complete in the 3 min that it took to record the spectrum, indicating that the decarboxylation of **4** to **5** is an enzyme-catalysed process. The results show that Cg10062 functions as a hydratase/decarboxylase using **11**, which represents a new activity in the tautomerase superfamily.

Deciphering the reaction mechanism of Cg10062

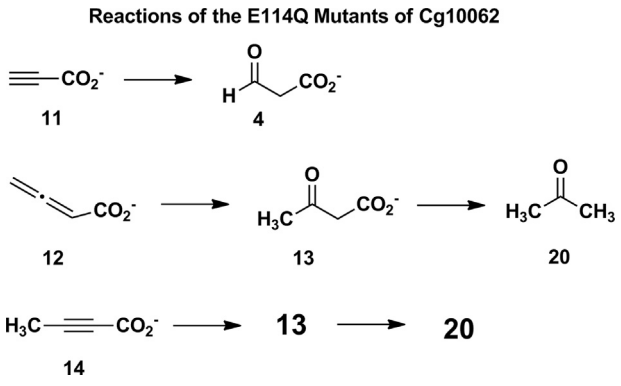
These experiments prompted two questions: does the reaction occur at the active site and does Cg10062 catalyse the decarboxylation of **4**, the presumed product



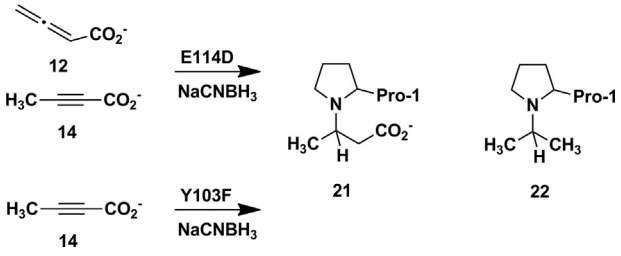
Scheme 5



Scheme 6



Scheme 7



Scheme 8

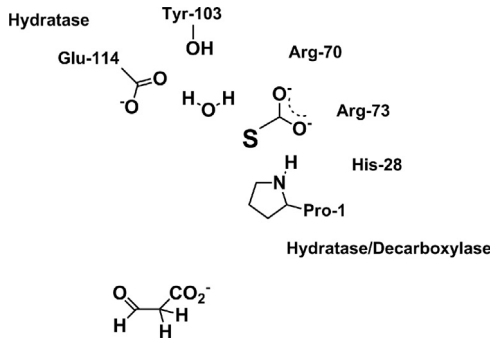
of hydration? Mutations of Pro-1 (P1A), Arg-70 (R70A), or both Tyr-103 and Glu-114 (Y103F/E114Q) greatly reduced the activity (over that observed for the wild type), indicating that the reaction occurs at the active site. Moreover, exogenous **4** was not decarboxylated by Cg10062, suggesting that the sequence of reactions (i.e., hydration followed by decarboxylation) likely occurs only when the enzyme is presented with **11**.

We next examined the scope of the reaction by incubating Cg10062 with the *cis* and *trans* isomer of 3-chloroacrylate (**2** and **3**), the allene, 2,3-butadiene (**12**, Scheme 4), and 2-butynoate (**14**). With **2**, Cg10062 functions as a hydratase/decarboxylase, although a poor one ($k_{cat}/K_m \sim 14\ M^{-1}\ s^{-1}$), generating a small amount of **4** (5%) and a larger amount of **5** (27%) after 48 min. A significant amount of **2** remained (68%) (after 48 min). With **3**, the reaction was slower ($k_{cat}/K_m \sim 1\ M^{-1}\ s^{-1}$) with small amounts of **4** (9%) and **5** (2%) being produced after 48 min. The decarboxylation of **4** to **5** observed in the reaction mixture containing Cg10062 and **3** is non-enzymatic. Interestingly, Cg10062 functions only as a hydratase using the allene (**12**) and 2-butynoate (**14**). Both were processed to acetoacetate (**13**), which likely results by the addition of water to C-3 followed by tautomerization of the product. There was no evidence for the enzyme-catalysed decarboxylation of **13** in the reaction with **12**, but there might be a trace amount of decarboxylation product in the reaction with **14**. The allene is a reasonably good substrate for Cg10062 ($k_{cat}/K_m \sim 5.1 \times 10^3\ M^{-1}\ s^{-1}$), but **14** is a poor one ($k_{cat}/K_m \sim 30\ M^{-1}\ s^{-1}$). After 3 min, NMR analysis shows that the conversion of **12** to **13** is nearly complete, whereas it takes 39 min to convert **14** to **13**.

cis-CaaD functions as a hydratase using **2**, **11** ($k_{cat}/K_m \sim 6.4 \times 10^3\ M^{-1}\ s^{-1}$, as noted above), and **12** ($k_{cat}/K_m \sim 8.7 \times 10^3\ M^{-1}\ s^{-1}$) (Schroeder et al., 2012). *cis*-CaaD processes **14** to **13**, (determined by ^{1}H NMR spectroscopy), but the rate is too slow to obtain kinetic parameters. *cis*-CaaD is highly specific for the *cis*-isomer and no hydration is seen with the *trans*-isomer (i.e., **3**) (Poelarends et al., 2004a).

Two mechanisms can be envisioned for the hydration/decarboxylation reaction of Cg10062 with **11** (Schemes 5 and 6). The first mechanism, analogous to that proposed for *cis*-CaaD in Scheme 2, involves direct hydration of the substrate by water (Scheme 5). The resulting product from the hydration of **11** (i.e., **4**) would then undergo decarboxylation. In this scenario, the water molecule is activated by the combination of Glu-114 and Tyr-103 while **11** is polarized by its interaction with Arg-70, Arg-73, and His-28. These actions produce **15**, which can tautomerize to **4**. Pro-1 can provide a proton at C-2 in the hydration step, the tautomerization step, or in both steps. Subsequently, **4** is decarboxylated in the active site to form the enol of **5**. Tautomeriztion produces **5**. This mechanism is at odds with the observation that the exogenously added **4** is not decarboxylated.

The second mechanism involves covalent catalysis using Pro-1 (Scheme 6). In this mechanism, Pro-1 adds to C-3 of **11** to form **16**, which can rearrange to the imine, **17**. Decarboxylation of **17** yields **18**, which can rearrange to the imine, **19**. Subsequent hydrolysis produces **5** and the free enzyme. The covalent catalysis mechanism can account for the observation that exogenously added **4** is not decarboxylated. However, the pK_a of Pro-1 is estimated to be 9.2, (based on a pH rate profile) (Poelarends et al., 2004b). The pK_a suggests that the prolyl nitrogen is largely cationic and unable to function as a nucleophile. This apparent discrepancy has two possible explanations: the pH rate profile does not reflect the actual pK_a of the prolyl nitrogen or the reaction proceeds using the small amount of enzyme with the prolyl nitrogen in the uncharged form. Reaction of the uncharged form with substrate perturbs the



Scheme 9

equilibrium so that an additional amount of enzyme has the prolyl nitrogen in the uncharged form. The reaction continues in this manner until all the substrate is processed.

Preliminary evidence supporting the covalent catalysis mechanism was obtained in studies of the E114Q, E114D, and Y103F mutants of Cg10062 (Schemes 7 and 8). Interestingly, these mutations cause a switch in some activities. For example, the E114Q mutant functions as a hydratase with **11**, producing only **4**, but as a hydratase/decarboxylase with **12** and **14**, producing a mixture of **13** and acetone (**20**, Scheme 7). With **11**, the E114Q mutant produces **4** (6.5%) and a small amount of **5** (0.5%) after 18 min. The small amount of **5** is likely due to the non-enzymatic decarboxylation of **4**. A large amount of unreacted substrate remains (93%). With **12**, the reaction is complete in 12 min. The reaction mixture consists of mostly **13** (83%) and a significant amount of **20** (17%). With **14**, the E114Q mutant produces **13** (0.8%) along with **20** (2.2%) after 90 min. A large amount of unreacted substrate remains (97%). Of these three reactions, the E114Q mutant is most efficient using **12**. Again, it was found that the E114Q mutant does not decarboxylate exogenously added **13**. This is indicated by the observation that an additional 15 min incubation period does not result in the further decarboxylation of **13** that is present after completion of the reaction using **12**.

The reactions of the E114D and Y103F mutants with **11**, **12**, and **14** were also examined. The E114D mutant functions as a hydratase/decarboxylase with **12** and **14** (producing a mixture of **13** and **20**), whereas the Y103F mutant functions as a hydratase/decarboxylase with **14**. For both **12** and **14**, the hydratase/decarboxylase activity of the E114D mutant is not as robust as that observed with the E114Q mutant. In contrast, the Y103F mutant shows modest hydratase/decarboxylase using **14** and produces a mixture of **13** (84%) and **20** (16%) after 6 min.

With these results in hand, incubation mixtures were set up containing the E114D or the Y103F mutant, substrate (E114D with **12** and **14** and Y103F with **14**), and $NaCNBH_3$ (Scheme 8). In a covalent catalysis mechanism, Pro-1 of the enzyme would form an imine bond with acetoacetate (**13**) and/or acetone (**20**) (analogous to **17** and **19**, respectively, in Scheme 6). The presence of $NaCNBH_3$ would reduce (and trap) the imine species as **21** (the reduced imine species resulting from **13** and enzyme) or as **22** (the reduced imine species resulting from **20** and enzyme). Mass spectral analysis of all mixtures showed both **21** (17,165 Da) and **22** (17,127 Da) in all mixtures (in addition to the unlabelled enzyme). The mass spectrum for the mixture containing Y103F, **14**, and $NaCNBH_3$ is shown in Figure 5. The two major

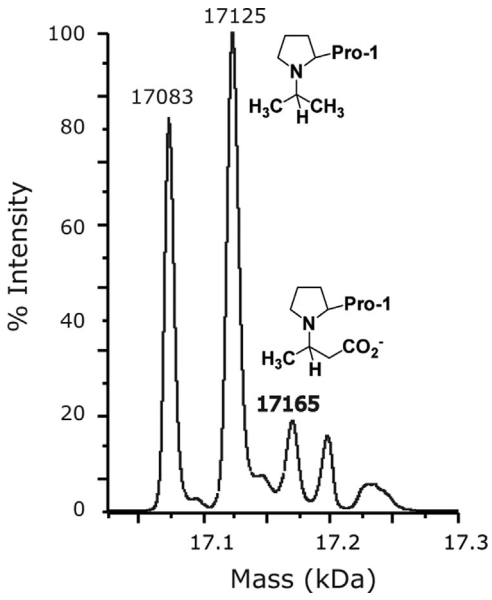


Figure 5 ESI-MS spectra of the Y103F mutant of Cg10062 incubated with **14** in the presence of $NaCNBH_3$. The signal at 17,083 Da corresponds to the unlabelled Y103F-Cg10062. The signals at 17,125 Da and 17,165 Da correspond to Y103F-Cg10062 covalently modified by the reduced imine of **22** and **21**, respectively.

signals correspond to the unlabelled Y103F mutant (17,083 Da) and **22** (17,125 Da). A smaller signal at 17,165 Da corresponds to **21**. Attempts to covalently modify these enzymes in the presence of exogenously added acetone or acetoacetate (and $NaCNBH_3$) were not successful. This observation suggests that **21** and **22** do not result from the reduction of an adduct formed between products (i.e., **13** and **20**, respectively) and enzyme. The remaining combinations of mutant and substrate (E114D with **11** and Y103F with **11** and **12**) were not subjected to trapping experiments or failed to trap the imine products.

The trapping experiments are consistent with covalent catalysis involving the nucleophilic attack of Pro-1 (in the wild-type enzyme) on the two substrates (**2** and **11**) that undergo a hydration/decarboxylation reaction (Scheme 6). The two other substrates (**12** and **14**) undergo only the hydration reaction with Cg10062. The mechanistic pathway that predominates might be related to the substrate's position in the active site with respect to the two water-activating residues (Glu-114 and Tyr-103) and Pro-1 coupled with the accessibility of C-3 (Scheme 9). Assuming the carboxylate groups of the four substrates interact with the 3 positively charged residues (Arg-70, Arg-73, and His-28), then C-3 of **2** and **11** might be accessible to Pro-1 facilitating covalent catalysis. The additional methyl group on **14** and the rigid allene system in **12** might limit flexibility and preclude the addition of Pro-1. There are other possible explanations, as well.

Conclusions

The proposed mechanism for the bifunctional hydratase/decarboxylase activity raises (at least) four questions. First, the pK_a of Pro-1, estimated to be 9.2, is not consistent with a proposed nucleophilic function. As noted above, this value is based on a

pH rate profile and might not reflect the actual pK_a value (Poelarends et al., 2004b). Efforts are underway to measure the pK_a of Pro-1 by ^{15}N NMR spectroscopy. Second, it is not possible to trap the imine as **21** or **22** in some reactions even though the imine should be present in "trappable" quantities. For example, using **12**, the E114Q mutant of Cg10062 generates **13** (83%) and **20** (17%) in 12 min. However, there is no evidence for **21** or **22** when the reaction is carried out in the presence of $NaCNBH_3$. Third, it is not clear why a mixture of **4** and **5** (15% and 85%, respectively) results in the reaction of **11** and Cg10062 because the reaction should go to completion with only **5** being present. Hydrolysis of **17** (Scheme 6) could release **4**. One possibility is that hydrolysis at this step represents a promiscuous activity. Finally, it is not clear what features of Cg10062 facilitate the decarboxylation of the imine. Along these lines, it is not clear why exogenously added **4** is not decarboxylated by the enzyme in that it can form a Schiff base with the prolyl nitrogen. All of these questions are under study.

The final question is whether the hydratase/decarboxylase reaction with **11**, the substrate with the highest value of k_{cat}/K_m ($\sim 1.8 \times 10^5\ M^{-1}\ s^{-1}$), has biological relevance. Acetylene compounds are well known in nature (e.g., calicheamicin and other enediyne antibiotics) and there are various reports concerning their transformation and degradation in bacteria. The genomic context for Cg10062 does not provide any clues about function because the genes in proximity have unknown functions or tentatively assigned functions that are not helpful. There are two reports in the literature involving **11**, but neither is informative. In the late 1950s an unidentified enzyme was isolated from *Pseudomonas* and reported to convert **11** to **4** (Yamada and Jakoby, 1958). In the early 2000 s, a *Pseudomonas putida* strain was isolated from rotten fruits with the ability to grow on **11** as a sole carbon source (Brecker et al., 2003). ^{1}H NMR spectroscopy following the biotransformation of **11** in cells showed the formation of **4** and **5**. There is also a report in the KEGG database suggesting a pathway for the conversion of **11** to **4**, and then the conversion of **4** to β-alanine (Kanehisa et al., 2002). The basis for this pathway is not entirely clear. However, β-alanine is converted to pantothenic acid in series of reactions in *C. glutamicum*, which is known for the overproduction of pantothenic acid (Merkamm et al., 2003).

We have previously proposed that CaaD and MSAD might have diverged from a common ancestral enzyme that catalysed both reactions (Poelarends et al., 2005; Almrud et al., 2005). This scenario was suggested by the observations that the two enzymes are structurally homologous, they catalyse hydration reactions using conserved groups, and they catalyse successive reactions in the same catabolic pathway. Cg10062 could be representative of the progenitor for the two enzymes. This possibility is currently being explored.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The research described in this paper was supported in part by the National Institutes of Health Grant GM-065324 and the Robert A. Welch Foundation Grant F-1334.

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